Appendix A

Synovial membrane is a vascular connective tissue that lines the inner surface of the capsule but does not cover the bearing surfaces. It consists of formed elements such as cells and fibers, together with intercellular material called matrix or ground substance. The connective tissue cells (synoviocytes) that are adjacent to the joint cavity are grouped together in one to three layers to form a relatively smooth surface from which a variable number of folds – villi - and fat pads project onto the joint cavity. Immediately subjacent to these surface cells is a capillary network. Scanning electron microscopy demonstrates characteristic undulations of the surface and separation of the synoviocytes. Two types of synoviocytes (A&B) have been described in a number of species. These types differ in their ultrastructure, and may represent different states of activity of the same cell type. The tissue immediately subjacent to these surface cells and capillary network may be fibrous, areolar, or fatty. It varies in thickness and contains fibroblasts, macrophages, mast cells, and fat cells as well as blood and lymphatic vessels and a few nerve fibers. If a synovial membrane is removed, a new synovial membrane may form from this underlying tissue or from the joint capsule.

The ground substance or matrix of any connective tissue when examined under the light microscope seems to be an amorphous substance. All ground substances contain complex compounds of high molecular weight, including mucopolysaccharides, a sulfate free compound termed hyaluronic acid. All mucopolysaccharides are complex asymmetric long-chain molecules that form viscous sols or even gels. They are attacked by a variety of enzymes usually called hyaluronidases. It is thought that basic units, probably disaccharides, are formed elsewhere and brought to connective tissue by the blood stream. In joints, synoviocytes synthesize these units into hyaluronic acid, probably chiefly by polymerization, that is, by a linking together of the basic units. This is an active energy-requiring process. It is not yet known, however, whether type A cells alone, or type B, or both, are responsible.

Formulation # 2:

10% Mannitol
10% Maltodextrin
0.1% Polysorbate 80

Formulation # 3:

10% Mannitol
0.1% Polysorbate 80

Formulation # 4:

10% Mannitol
0.1% Polysorbate 80
2% serum albumin

Formulation # 5:

5% Mannitol
5% Glycine
0.1% Polysorbate 80

Each stabilizer solution was mixed with an equal volume of synovial fluid, and 2 ml was filled into 5 ml vials. The product was freeze-dried by freezing overnight at -40°C, followed by drying for 48 hours at a shelf temperature of -35° C and 12 hours at 25° C.

Product vials were stored at 5° C, -20° C, and -80° C (control.) Each formulation was tested about every two weeks for a total of ten weeks. The freeze-dried solid was reconstituted with 1 ml of water prior to testing.

Mucin Clot Test

The reconstituted formulation (100 µL) was added, using an Eppendorf pipette, to 1 mL 10% v/v acetic acid in a 2 cm radius petri dish. The test is based on visual assessment of the morphology of the resulting mucin clot using criteria established by Ropes and Bauer (1953). The clot was compared with pictures from the above reference rating the clot as a, b, c, or d:

a. A tight clump surrounded by a clear solution
b. A soft mass not as tightly clotted as category "a"
c. A clot that is dispersed over a relatively large area with no distinct shape. Some discrete pieces are dispersed within the clot area
d. A few ropy strands in a cloudy solution. No distinct clot is formed

Results and Discussion

Mucin clot test data from the stability study are shown in Tables I-III for 5° C, -20° C, and -80° C, respectively. Duplicate results are recorded where available. A second test was carried out using reconstituted solid from the same vial. The subjective nature of the mucin clot, test is a major limitation of the study, as can be seen from the data in these tables. Every attempt was made to use consistent technique; that is, attempting to expel the reconstituted solid at a constant rate into the acetic acid solution and placing the tip of the pipette just below the surface of the liquid.

Based on the SOC data (Table I), formulations 1 and 2 appear to be the best, even though clots as poor as "d" were observed at times during the stability study. Formulation 5 may be as good as formulations 1 and 2 however, considering the variability of the test method. Formulations 3 and 4 yielded, the poorest clots initially, and they remained the poorest clots throughout the study. The variability in the data do not allow a definitive conclusion as to whether there is a loss in the quality of the clot during storage at 5° C.

Table II summarizes the -20° C data. Again, formulations 1 and 2 appeared to result in the best clots initially as well as at the end of the study, but clot quality as low as "d" was observed for formulation 1 and "c-d" for formulation 2. Formulation 5 was next, and formulations 3 and 4 were the worst. The data in Table II do not allow a clear conclusion as to whether there is a significant decrease in clot quality during storage of the freeze-dried solid.

Storage at -80° C would not be expected to result in loss of activity during storage. However, as shown in Table III, all of the formulations showed poorer clots at the end of the study than at the beginning. The reason for this is not known at present.

Reconstituted stability data are summarized in Table IV, where reconstituted solids from the last time interval were examined within minutes after reconstitution and again after 19 hours at room temperature. There is a significant loss of clot quality, although the test method used does not allow a quantitative assessment. The data do support the conclusion that the product should be used as soon as practical after reconstitution.

Conclusions

Despite the limitations of the test method, the stability data at 5° C and -20° C support the conclusion that formulations 1 and 2 provide the best product, although no firm conclusions can be drawn as to whether there is a significant loss of activity during storage. Polysorbate 80 seems to offer no particular advantage, since it was present in both "goody' and "bad" trial batches. Likewise, human serum albumin does not appear to offer any advantage. Maltodextrin does seem to help, but formulations 1 and 2 were only marginally better than formulation 5, which contains Mannitol, Glycine, and Polysorbate 80.

Reconstituted stability data show that the product should be used as soon as practical after reconstitution.